OPTIMIZATION OF CAPILLARY ISOELECTRIC FOCUSING FOR RAPID ANALYSIS OF BIOLOGICAL MOLECULES

by

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Abstract

Currently in the field of bioanalysis, a large amount of information must be obtained and processed in a timely manner. Despite its exceptional separation power, progress using capillary isoelectric focusing (cIEF) as an analytical tool has been stagnating in this rapidly advancing field without significant means to shorten analysis time. To address this limitation, 30-μm internal diameter capillaries were used to achieve full pH range (pH 3-10) cIEF within 15 minutes. The benefits of using narrow diameter capillaries are demonstrated by comparing with results obtained by using 50-μm internal diameter capillaries. Optimization of additional experimental parameters such as presence of cathodic spacer, concentration of anticonvective media, and focusing time helped to achieve much shorter analysis time. While sensitivity is sacrificed in the process of reducing analysis time with the change of some parameters, such loss can be reasonably accommodated given the exceptional sensitivity of cIEF.

Prior to achieving rapid cIEF with narrow diameter capillary, reproducibility of cIEF is examined by 60 consecutive experiments on a single capillary. Despite measures taken to ensure minimal analyte adsorption and protein precipitation, migration times of analytes increased in subsequent experiments until the capillary was conditioned by extended rinsing. Unfortunately, conditioning of the capillary degrades the internal coating and shortens its lifespan to approximately 50 consecutive experiments when conditioned every 10 experiments. Notwithstanding substantial variation in migration times, calculated pI values of the sample protein, myoglobin, are accurately and precisely determined
throughout repeated experiments. Capillary isoelectric focusing can therefore be relied upon to separate and identify biological analytes such as proteins.
Preface

The results included in this thesis and the writing were completed entirely the author, Alexis Lee. The design of the research was accomplished with advice from Dr. David Chen. The 30-μm neutral capillary was generously provided by Beckman Coulter, Brea, California, USA, before its commercial launch.
# Table of Contents

Abstract ........................................................................................................... ii  
Preface ............................................................................................................... iv  
Table of Contents ............................................................................................. v  
List of Tables ..................................................................................................... vii  
List of Figures ................................................................................................... viii  
List of Symbols and Abbreviations ................................................................. x  
Acknowledgements ........................................................................................ xii  
Dedication .......................................................................................................... xiii  

Chapter 1: INTRODUCTION TO CAPILLARY ISOELECTRIC FOCUSING IN BIOANALYSIS .................................................................................................................. 1  
1.1 Two-dimensional separation of complex biological mixtures ............ 2  
1.2 Capillary isoelectric focusing ................................................................. 4  
  1.2.1 Emergence of capillary isoelectric focusing .................................. 4  
  1.2.2 Experimental setup of capillary isoelectric focusing ................. 6  
  1.2.3 Mechanism of separation .............................................................. 8  
  1.2.4 Mobilization .................................................................................... 14  
  1.2.5 Detection ....................................................................................... 18  
1.3 Research objectives ................................................................................. 22  
  1.3.1 Reproducibility of capillary isoelectric focusing ....................... 22  
  1.3.2 Optimization for rapid capillary isoelectric focusing ............. 23  

Chapter 2: REPRODUCIBILITY OF CAPILLARY ISOELECTRIC FOCUSING IN 50-μm INNER DIAMETER NEUTRAL CAPILLARY ..................................................... 24  
2.1 Introduction .............................................................................................. 25  
2.2 Experimental ............................................................................................ 26  
  2.2.1 Instrumentation ............................................................................ 26  
  2.2.2 Chemicals and materials ............................................................. 27  
  2.2.3 Preparation of pI standards ......................................................... 27  
  2.2.4 Methods ....................................................................................... 27  
2.3 Results and discussion ............................................................................ 29  
  2.3.1 Peak identity and determination of myoglobin pI ..................... 29  
  2.3.2 Reproducibility ................................................................. 30  
2.4 Conclusions .............................................................................................. 36  

Chapter 3: OPTIMIZATION FOR RAPID CAPILLARY ISOELECTRIC FOCUSING WITH 30-μm INNER DIAMETER NEUTRAL CAPILLARY ........................................... 38  
3.1 Introduction .............................................................................................. 39  
3.2 Experimental ............................................................................................ 42
Chapter 3: INSTRUMENTATION

3.2.1 Instrumentation ................................................................. 42
3.2.2 Chemicals and materials ..................................................... 42
3.2.3 Preparation of pI standards .................................................. 43
3.2.4 Methods ............................................................................. 43

3.3 RESULTS AND DISCUSSION ...................................................... 44
3.3.1 Arginine as a cathodic spacer ............................................. 44
3.3.2 Mobilization ....................................................................... 48
3.3.3 Anti-convective focusing media ......................................... 52
3.3.4 Focusing time ..................................................................... 55
3.3.5 Compatibility with mass spectrometry ................................. 56

3.4 CONCLUSIONS ........................................................................ 57

Chapter 4: CONCLUSIONS ............................................................... 59

4.1 REALIZATION OF RESEARCH OBJECTIVES .......................... 60
  4.1.1 Reproducibility of capillary isoelectric focusing .................. 60
  4.1.2 Optimization for rapid capillary isoelectric focusing .......... 61

4.2 FUTURE RESEARCH DIRECTIONS ......................................... 62
  4.2.1 Coupling with mass spectrometry ...................................... 62
  4.2.2 Rapid targeted screening ................................................... 63

4.3 CONCLUDING REMARKS ......................................................... 63

Bibliography ................................................................................... 65
List of Tables

Table 2.1 The average electroosmotic mobility of three replicate measurements and migration times of the pI 7 marker. Electroosmotic mobility (\[\mu_{eo}\]) was measured at the start of each set of 10 runs, before run \(n\) for the row. Migration time, \(t_n\), refers to that of the run number indicated by the leftmost column while \(t_{n+9}\) refers to the run that is 9 runs after the run number, \(n\). ..................................................................................................................................................34

Table 3.1 Peak heights and resolution of the peptide pI standards with three modes of mobilization: chemical, pressure, and pressure-assisted chemical mobilizations.........50

Table 3.2 Peak areas of five pI standards as a function of glycerol concentration in the sample mixture..................................................................................................................................................55

Table 3.3 Peak areas of five pI standards with varying focusing time. Chemical mobilization was triggered 7 or 12 minutes after the start of analysis. ......................................................................................................................56
List of Figures

Figure 1.1 Schematic of a simple capillary isoelectric focusing setup. The orange wire represents fused silica capillary and the blue wire represents electrodes and electric circuit. High voltage supply is represented by the blue box with HV.................................................7

Figure 1.2 Structures of glutamic acid as a function of pH.........................................................9

Figure 1.3 Computer simulation of 140 carrier ampholytes and 3 dyes (pI 6.6, 7.4, and 8.6) in the absence of electrolyte solutions. The arrows indicate the location of the dyes. A: concentration profiles of the ampholytes across the entire length of the column. B: a magnified section of the column. Adapted with permission from Mao and others’ work28. Copyright © 2000 American Chemical Society.................................................................13

Figure 1.4 Detector response to pI 6.6, 7.4, 8.6 dyes at various time intervals as indicated by y-axis inset. A: computer simulated response. B: experimental data with whole column imaging. Reprinted with permission from Mao and others’ work29. Copyright © 2000 American Chemical Society................................................................................................................14

Figure 1.5 Computer simulated concentrations, pH, and conductivity during chemical mobilization in a 3-cm capillary, boundaries of which are indicated by vertical dotted lines. The bottom trace of overlapping Gaussian peaks illustrate migration of carrier ampholytes while four sharp peaks labelled Hbs are hemoglobin isoforms (augmented by 100-fold and offset by 25 mM) show positions of the analytes. Anolyte of 100-mM H3PO4 and cathodic mobilizer of 40-mM NaOH and 20-mM HCl are represented by appropriately labelled solid and dotted lines. Another solid trace labelled pH illustrates the pH gradient while top trace labelled cond. shows conductivity. Inset: concentration of chloride in the space occupied by carrier ampholytes. Reprinted with permission from Thormann and Mosher’s work34. Copyright © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim........................................................................................................17

Figure 2.1 A representative electropherogram of a complete cIEF experiment using pI 3-10 carrier ampholytes and 5 peptide pI standards at pI 10.0, 9.5, 7.0, 5.5, and 4.1. Two myoglobin isoforms were observed as labelled in the inset. Inset: magnified electropherogram from 40 to 45 minutes........................................................................................................29

Figure 2.2 Migration times of standard pI markers (dotted lines) and calculated pI values of two myoglobin isoforms (solid lines) over 60 replicate runs. Fresh sample and electrolyte solutions were used every 5 runs and the capillary was conditioned every 10 runs. Disconnects on pI 4.1 and pI 10.0 traces are due to analyte peaks being outside the pre-set analysis time window.....................................................................................................30

Figure 2.3 Partial electropherograms approximately from pH 7.5 to 4.5, showing myoglobin isoforms and pI standards 7.0 and 5.5. The order of each experiment among the 60 replicate runs in Figure 2.2 is indicated on the left side. ........................................................................................................31
Figure 3.1 Electropherograms of three peptide pI markers (pI 10.0, 9.5, and 7.0) with varying concentrations of arginine in the sample mixtures. The sample mixtures were composed of 50% v/v cIEF gel. Mobilization started at 15 minutes. A: 0% (offset by 0.03 Au); B: 1% (offset by 0.02 Au); C: 3% (offset by 0.01 Au); and D: 8% v/v of 500 mM arginine.

Figure 3.2 Electropherograms of four peptide pI standards (pI 10.0, 7.0, 5.5, 4.1) with three different mobilization methods. The sample mixture contained 50% v/v cIEF gel with no ARG. Mobilization started at 15 minutes. From bottom: chemical mobilization, pressure mobilization (offset by 0.01 Au), and pressure-assisted chemical mobilization (offset by 0.02 Au). The pressure applied at the inlet for pressure and pressure-assisted chemical mobilization was 1 psi.

Figure 3.3 Electropherograms of five peptide pI standards (pI 10.0, 9.5, 7.0, 5.5, 4.1) in varying concentrations of glycerol. Pressure-assisted chemical mobilization started at 12 minutes with 1 psi applied at the inlet. A: 5% (offset by 0.03 Au); B: 10% (offset by 0.02 Au); C: 15% (offset by 0.01 Au); and D: 20% v/v glycerol in water as separation media.
List of Symbols and Abbreviations

2DGE Two-dimensional gel electrophoresis
A Cross-sectional area
ARG Arginine
C Concentration of a component
CAAs Carrier ampholytes
CCD Charge coupled device
CE Capillary electrophoresis
cIEF Capillary isoelectric focusing
CM Chemical mobilization
$C_m$ Conductance of a medium
D Diffusion coefficient
DMF N,N-dimethylformamide
E Electric field
EOF Electroosmotic flow
ESI Electrospray ionization
FC Fluorocarbon
H Plate height
HPLC High-performance liquid chromatography
I Electric current
ID Inner diameter
IDA Iminodiacetic acid
IEF Isoelectric focusing
ITP Isotachophoresis
K Distribution coefficient of analyte
$k_d$ Rate constant of desorption
LC Liquid chromatography
$L_T$ Total length of capillary
MALDI Matrix-assisted laser desorption/ionization
MS Mass spectrometry
MS/MS Tandem mass spectrometry
Myo Myoglobin
n Peak capacity
OD Outer diameter
PB7 Phosphate buffer at pH 7
PCM Pressure-assisted chemical mobilization
pI Isoelectric point
PM Pressure mobilization
q Charge of ion
R Radius of solvated ion
$R_s$ Resolution
RSD Relative standard deviation
r Radial distance from the centre of capillary
$r_c$ Capillary radius
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<tr>
<td>TOF</td>
<td>Time-of-flight</td>
</tr>
<tr>
<td>$t_n$</td>
<td>Migration time of a peak</td>
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<tr>
<td>UPLC</td>
<td>Ultra-performance liquid chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>$V_{prog}$</td>
<td>Applied potential</td>
</tr>
<tr>
<td>$v_m$</td>
<td>Mobilization velocity</td>
</tr>
<tr>
<td>$w_n$</td>
<td>Width of a peak</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Viscosity</td>
</tr>
<tr>
<td>$\mu_{eo}$</td>
<td>Electroosmotic mobility</td>
</tr>
<tr>
<td>$\mu_{ep}$</td>
<td>Electrophoretic mobility</td>
</tr>
</tbody>
</table>
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Dedication

To Gio, my sunshine.
Chapter 1: INTRODUCTION TO CAPILLARY ISOELECTRIC FOCUSING IN BIOANALYSIS
1.1 Two-Dimensional Separation of Complex Biological Mixtures

In pursuit of a deeper understanding of living organisms, intense research efforts have been dedicated to analysis of biological molecules. Since the successful completion of the Human Genome Project in 2003, proteomics has largely replaced genomics at the centre stage of bioanalysis. Proteomics is a field dedicated to large-scale analysis of proteins and their expressions at a cellular level to characterize biological processes\(^1\). Its relevance to prominent research areas such as molecular medicine, cellular functions, and evolutionary genetics has placed it at the hub of vigorous research activity in recent years.

Although the field of proteomics arguably dates back to the 1970s when proteomic databases had begun to be assembled, much of the progress only occurred after recent developments in quantitative analytical techniques. The necessity for analytical advancements is especially evident when considering the complexity of the human proteome, which is comprised of over 10 million molecular forms including protein fragments and their various modifications. The vast dynamic range of the human proteome also poses great difficulty for quantitative analysis as signals from abundant proteins often obscure signals from those of low abundance. Furthermore, detection of low abundance proteins after extensive extraction procedures is difficult, especially in traditional techniques such as gel electrophoresis. To address these challenges, faster and more sensitive analytical methods and instrumentation have been emerging at a rapid pace.

To analyse such complex samples, a multi-dimensional approach is crucial. With any single analytical tool, there is a limit to its peak capacity which is defined as the number of
peaks that can be contained into the physical separation space at a specified resolution\(^2\).

Peak capacity, \(n\), is expressed in an equation as the following:

\[
n = \frac{L_T}{4\sigma R_s} \tag{Eq. 1.1}
\]

where \(L_T\) refers to the total length of separation column; \(\sigma\), standard deviation of a peak; and \(R_s\) specified resolution. When two analytical techniques with orthogonal separation parameters are combined, peak capacity increases thusly:

\[
n = n_1 n_2 \tag{Eq. 1.2}
\]

where \(n_1\) and \(n_2\) refer to peak capacity of each separation. The key to multi-dimensional separation in this fashion is the orthogonality of separations: analytes must be separated based on distinct properties, given that combining these separation methods does not adversely affect resolution. For example, analytes in two-dimensional gel electrophoresis (2DGE) are separated based on their size-to-charge ratio and isoelectric points (pl). Other examples of two-dimensional separation include liquid or gas chromatography with mass spectrometry (LC-MS or GC-MS) and capillary electrophoresis with MS (CE-MS). Moreover, with increasingly complex samples demanded by current applications, it is becoming more common to see higher-dimensional separations, such as LC-CE-MS\(^3\)-\(^4\). Peak capacity increases proportionately with each additional dimension of separation. Therefore, to adequately separate complex samples prevalent in modern biological analyses, it is important to have available multiple high-resolution separation methods with orthogonal properties.
1.2 Capillary Isoelectric Focusing

1.2.1 Emergence of capillary isoelectric focusing

One of the defining moments in multi-dimensional separation occurred in 1975, when O’Farrell combined two orthogonal separations to resolve more than 1000 compounds in two-dimensional gel electrophoresis (2DGE). There have been several reports of 2DGE prior to O’Farrell’s work. However, these are not considered two-dimensional separations by the current definition, as the protein fragments were separated by size-to-charge ratio in two spatial dimensions rather than by using two independent parameters. Two-dimensional separation by two independent properties can increase peak capacity while providing additional information on the protein fragments. Isoelectric focusing (IEF), as O’Farrell discovered, is an excellent additional dimension of separation for analysis of proteins as it separates proteins with high resolution based on their isoelectric point (pI). Isoelectric point is a physical parameter, defined as the pH at which protein becomes neutral; both the sequence of amino acids and the structure of the folded state contribute to its pI. Furthermore, many post-translational modifications alter the pI by changing the charge state of the protein and IEF has been successful in differentiating between select modifications. In addition to its exceptional capability for separation, isoelectric focusing can be used to determine the pI of an unknown protein, thereby providing information on its identity and function.

The concept of separation by isoelectric focusing has been around for almost a century. However, it was only in 1961 when Svensson was able to demonstrate proof-of-concept for successful separation of proteins in liquid media. Since then, there have
been continual improvements on stability, reproducibility, and resolution of this technique; some highlights include development of polyacrylamide gel as separation medium\textsuperscript{9}, incorporation of sodium dodecyl sulfate gel electrophoresis for two-dimensional separation\textsuperscript{5}, and invention of immobilized pH gradients\textsuperscript{16}. Capillary isoelectric focusing (cIEF) is yet another step toward high-throughput quantitative analysis of proteins with promise of easy automation, short analysis time, and online coupling with mass spectrometry\textsuperscript{17}.

A fused silica capillary is well-suited for electric field based separations due to fast dissipation of Joule heating and reduced convection when high voltage is applied\textsuperscript{18}. The ability to apply high voltage is especially advantageous in isoelectric focusing as it is one of the two experimental parameters to improve resolution\textsuperscript{19}:

\[
\Delta pI = 3 \left( \frac{D \frac{d(pH)}{dx}}{E \left( - \frac{d\mu}{dpH} \right)} \right)^{\frac{1}{2}}
\]

(Eq. 1.3)

where \(\Delta pI\) refers to the smallest resolved difference in pl; \(D\), the diffusion coefficient of the analyte; \(x\), the distance along the capillary; \(\mu\), the mobility of the analyte; and \(E\), the applied electric field. Since the only controllable variables are \(dpH/dx\) and \(E\), it is important to be able to apply strong electric field for optimum resolution. Another great advantage of cIEF is its easy adaptability to an existing capillary electrophoresis (CE) system to enable automated sequencing. This is a monumental milestone on the way to high-throughput analyses, as an investigator can utilize multiple instruments to increase throughput by several folds. It also improves reproducibility because human errors are minimized.
Capillary isoelectric focusing is not without its share of challenges. Bare fused silica contains exposed silanol groups on the surface which become deprotonated at pH greater than 3. The excess negative charges from the silanoxide promote formation of electric double layer, which generates electroosmotic flow (EOF) toward the cathode when electric field is applied across the capillary. This phenomenon, while useful in capillary electrophoresis, is often detrimental in cIEF, since proteins cannot be focused in the correct pH region before being swept away by EOF. Additionally, protein adsorption due to electrostatic interaction with the capillary wall can lead to peak broadening and reduced sample recovery. In an effort to overcome these challenges, various coating methods have been proposed. These coatings have three major roles: to suppress EOF for successful focusing of pH zones, to minimize protein adsorption, and to remain intact across the pH range over many runs. While coatings that meet the three criteria are available, they are often difficult to prepare or costly if purchased from a commercial source. Moreover, they deteriorate after repeated exposure to high-pressure rinsing during experiments and must be replaced often.

In the following sections, an overview of capillary isoelectric focusing as a separation technique is described. It will provide a context in which the objectives of this thesis are rationalized. Setup of a typical experiment and mechanism of separation will be introduced, as well as various mobilization methods.

1.2.2 Experimental setup of capillary isoelectric focusing

Although a simple capillary isoelectric focusing experiment can be set up without great feat of engineering, most commercially available systems offer more than the bare
backbone by including software control, automation capabilities, and precise management of experimental parameters. When stripped down to the essential components, a typical setup is comprised of a fused silica capillary with its either end immersed in inlet and outlet vials containing electrolyte solutions, which in turn are connected to high voltage supply via metal electrodes (Figure 1.1). Depending on the direction of migration, either anode or cathode can be the inlet. In the figure and the rest of the section, inlet will be designated as the anode as it is conventionally set up in this fashion. The anolyte and catholyte are solutions of weak acid and base at the anodic and cathodic ends of the capillary, respectively. The electrolysis half-reactions in anolyte and catholyte complete the electric circuit as shown in Figure 1.1.

$$2H_2O(l) \rightleftharpoons O_2(g) + 4H^+_{(aq)} + 4e^-$$  
$$4H_2O(l) + 4e^- \rightleftharpoons 2H_2(g) + 4OH^-_{(aq)}$$

Figure 1.1 Schematic of a simple capillary isoelectric focusing setup. The orange wire represents fused silica capillary and the blue wire represents electrodes and electric circuit. High voltage supply is represented by the blue box with HV.

After adequate conditioning and rinsing of the capillary at the beginning of an experiment, a sample mixture is injected by pressure at the inlet to fill the entire capillary.
A sample mixture is composed of analytes dissolved in a low concentration carrier ampholytes (CAs) solution. Then high voltage typically in the range of 10 to 30 kV is applied to generate pH gradient and to focus the analytes. If slow EOF is present, the analyte will focus and migrate with EOF simultaneously, a process known as one-step cIEF. In two-step cIEF, EOF is sufficiently suppressed, and an additional mobilization step must follow to drive the focused zones toward optical detector or mass spectrometer located at or near the outlet. Since two-step cIEF has greater relevance to this thesis, suppressed EOF is assumed in the following sections.

1.2.3 Mechanism of separation

Amino acids are zwitterions at physiological pH: the N-terminus is protonated while the C-terminus is deprotonated. Additionally, certain residues contain acidic and basic functionalities with their specific $pK_a$ values and may be charged. The charge of the amino acid as a whole depends on the pH of the environment. The pH at which an amino acid becomes neutral, $pI$, is determined by the following formula:

$$pI = \frac{pK_1 + pK_2}{2}$$

(Eq. 1.4)

where $pK_1$ and $pK_2$ are the negative logarithms of the acid dissociation constants of the singly positive and neutral forms, respectively. For glutamic acid in Figure 1.2, those would be 2.16 and 4.29, yielding 3.22 as the $pI$. With proteins, theoretical calculation of $pI$ becomes much more complex due to structural considerations, but the zwitterionic nature of the molecule remains the same. A protein with a specific folded structure and post-translational modifications will have a unique $pI$ value that can be experimentally
determined. This information, in turn, can be used in the future to elucidate structure and modifications of proteins.

![Figure 1.2 Structures of glutamic acid as a function of pH](image)

When a protein is placed in a pH gradient under electric field, its direction of migration depends on its location along the gradient. When the pH is lower than its pI, it is positively charged and migrates toward the cathode; when the pH is higher than its pI, it is negatively charged and migrates toward the anode. These bidirectional forces eventually focus the proteins to a zone where its pI is equal to the pH, because there is no electrophoretic migration when it is neutral.

In cIEF, a solution of carrier ampholytes (CAs) mixed with the analytes is injected into the capillary. Commercially available solutions of carrier ampholytes are proprietary blends of high molecular-weight (approximately 200-1200 Da) polymers with oligo-amino backbone and acidic or basic functional groups. The main function of CAs is to generate a linear pH gradient under applied electric field. This is achieved by numerous ampholytes with minutely different isoelectric points, which are distributed somewhat evenly across the pH range of interest. The resolution of cIEF depends heavily on the buffering capacity and the narrowness of buffering region of each ampholyte species.

The theory of pH gradient formation with carrier ampholytes has been established in two important articles that Harry Svensson wrote in 1961–1962. A brief overview of the theory is explained, following the derivations by Svensson. After a solution of CAs is
injected into the capillary during cIEF, each end of the capillary is submerged in anolyte and catholyte solutions. Then high voltage is applied to the electrodes to generate electric field across the length of the capillary. Some hydrogen cations from anolyte enter the capillary by diffusion and electrophoresis, while hydroxide anions from catholyte enter from the cathodic end. These ions will charge ampholytes at either end of the capillary with positive and negative charges, respectively. Under electric field, the positively charged ampholyte ions migrate towards the cathode and the negatively charged ampholyte ions migrate towards the anode. Partial pH gradients start to form at the ends of the capillary, as there is higher density of cations and anions the closer it is to anode and cathode. Along these partial pH gradients, cationic ampholytes lose their positive charge and anionic ampholytes their negative charge as they find themselves in the pH environment, which is equal to their pI values. A neutral ampholyte does not migrate by electrophoresis, so it becomes immobile at its pI while buffering the pH of the environment. As the pH gradient extends towards the centre of the capillary, the neutral ampholyte may become charged again and start migrating. This process is repeated until a pH gradient is formed throughout the entire capillary with each ampholyte stationed near its pI.

Once the pH gradient has formed, assuming there is no electroosmotic flow, solution inside the capillary is at a steady state: there is no net mass flow into or out of the capillary. Carrier ampholytes and analytes remain at their pI along the pH gradient until the system is disturbed by external influences. While this seems intuitive at first, closer examination of the behaviour of ampholytes at their pI reveals some finer points to consider. In a solution of pure acidic ampholyte at its pI (i.e. pI is lower than 7), there exist equal concentrations of hydrogen ion and ampholyte anion. Although the concentration of hydrogen ion is close to
the negative logarithm of the \( p_l \), the existence of equal concentration of the ampholyte weak base raises the \( \text{pH} \) of the solution slightly higher than its \( p_l \). Similarly, the \( \text{pH} \) of a pure basic ampholyte solution would be slightly lower than its \( p_l \). Therefore, the ampholyte solution has a net charge: positive if its \( p_l \) is greater than 7, and negative if its \( p_l \) is less than 7. This is in conflict with the idea of \( \text{pH} \) gradient in steady state, as the net charge prompts the ampholyte to migrate electrophoretically. However, the steady state of \( \text{pH} \) gradient is corroborated by two observations: (1) electric current drops as focusing progresses until it reaches a low plateau, which signifies minimal ion movement; and (2) after sufficient focusing, analytes become stationary and the electropherogram remains static, except for protein precipitation\(^{26-27}\). To resolve this contradiction, Svensson suggested diffusion as the counter flow to the electrophoretic movement resulting from \( \text{pH} \)–\( p_l \) discrepancy\(^{12}\). This electrophoresis-diffusion balance is expressed in an equation as follows:

\[
\frac{C \mu_{ep} I}{AC_m} = D \frac{dC}{dx} \tag{Eq. 1.5}
\]

where \( C \) represents concentration of an ampholyte; \( \mu_{ep} \), electrophoretic mobility; \( I \), electric current; \( A \), cross-sectional area; \( C_m \), conductance of the medium; \( D \), diffusion coefficient; and \( x \), separation distance. The left side of the equation represents electrophoretic migration, whereas the right side of the equation represents diffusion. An analytical solution of (Eq. 1.5) with constant \( C_m \) and \( p \), defined in (Eq. 1.7), for an ampholyte in the capillary is given below:

\[
C = C_0 e^{-\frac{p_l x_0}{2AC_m D}} \tag{Eq. 1.6}
\]
where \( C_0 \) represents the concentration maximum and \( x_0 \) is separation distance defined as being equal to 0 at \( C_0 \). The variable \( p \) is defined as the negative derivative of electrophoretic mobility with separation distance:

\[
p = -\frac{d\mu_{ep}}{dx_0} = -\frac{d\mu_{ep}}{d(pH)} \frac{d(pH)}{dx_0}
\]

(Eq. 1.7)

The solution in (Eq. 1.6) describes concentration gradient of the ampholyte with a Gaussian distribution. This mathematical solution agrees with empirical considerations of ampholyte behaviour. The pH–pI discrepancy described previously leads to formation of a concentration gradient towards the anode for acidic ampholytes and towards the cathode for basic ampholytes. Since there is a finite amount of the ampholyte, the concentration gradient has a maximum where the derivative of concentration with respect to separation distance \( (dC/dx) \) becomes zero. Application of this observation to (Eq. 1.5) concludes that there is neither electrophoretic nor diffusional migration at concentration maximum. Lack of electrophoretic migration signifies that the concentration maximum of an ampholyte must be at its pI, where it becomes neutral. A corollary of this conclusion is the contamination of the ampholyte by the adjacent ampholyte. This is because on the electrode-side of the maximum (i.e. anodic for acidic ampholytes and cathodic for basic ampholytes), there must exist decreasing concentration gradient away from the maximum. The ampholyte species on the electrode-side of the maximum must also possess a net charge that enables them to electrophoretically migrate towards the maximum. It follows logically that the pH environment on the electrode-side of the maximum must be more acidic than the pI for acidic ampholytes and more basic for basic ampholytes. As the pure ampholyte solution proposed earlier cannot account for the existence of such species, they
must come from contamination by adjacent, more acidic or basic ampholytes, respectively. The resulting distribution of carrier ampholytes is a series of bell-shaped curves, each with its concentration maximum at its pI and overlapping with adjacent ampholytes. This conclusion is corroborated by several excellent computer simulations\textsuperscript{28-31}, an example of which is shown in Figure 1.3.

![Figure 1.3](image)

Figure 1.3 Computer simulation of 140 carrier ampholytes and 3 dyes (pI 6.6, 7.4, and 8.6) in the absence of electrolyte solutions. The arrows indicate the location of the dyes. A: concentration profiles of the ampholytes across the entire length of the column. B: a magnified section of the column. Adapted with permission from Mao and others’ work\textsuperscript{28}. Copyright © 2000 American Chemical Society.

Analytes of cIEF, which are amphoteric species with their own pI values, behave similarly to carrier ampholytes. They also materialize as Gaussian curves centred at its pI while overlapping with adjacent carrier ampholytes (Figure 1.3B). Focusing of analytes occurs simultaneously with the formation of pH gradient by the same mechanism as carrier ampholytes. As partial pH gradients start forming at either ends of the capillary, analytes migrate toward their pI along these partial gradients. Since the partial gradients only encompass small portions of the solution, the intensity of analyte peaks at this point is relatively low. As pH gradient forms across the entire capillary, analyte peaks from the ends of the capillary migrate towards the new location of its pI along the gradient, all the while growing in intensity, until they are focused. Computer simulated and experimental
observation of this double focusing effect is demonstrated in Figure 1.4. Once focused, the analyte peaks become sharper and more intense under continued electric field. Capillary isoelectric focusing is a technique that often produces high-resolution separation with sharp peaks because broadening of analyte peaks by diffusion is countered by bidirectional electrophoretic migration towards the centre of the analyte peak.

![Figure 1.4](image.png)

Figure 1.4 Detector response to pI 6.6, 7.4, 8.6 dyes at various time intervals as indicated by y-axis inset. A: computer simulated response. B: experimental data with whole column imaging. Reprinted with permission from Mao and others’ work\textsuperscript{28}. Copyright © 2000 American Chemical Society.

1.2.4 Mobilization

The most common type of detector for capillary isoelectric focusing is a single-spot detector positioned near or at the outlet of the capillary (discussed in sections 1.2.5.2 and 1.2.5.3). As the analytes are stationary at their isoelectric points at the end of focusing, they
must be mobilized toward the detector. Two methods of mobilization most often employed are hydrodynamic mobilization and electrophoretic mobilization.

1.2.4.1 Hydrodynamic mobilization

The focused zones are most easily mobilized by applying pressure\(^{20}\) or vacuum\(^{32}\) to generate hydrodynamic flow. In both cases, linear migration with \(pI\) is accomplished because this mobilization method does not discriminate against \(pI^{18}\). The most prominent disadvantage of hydrodynamic mobilization is reduced resolution caused by the laminar flow profile. This laminar flow profile is the result of the balance between the forward pressure and viscous forces that resist it. Since viscous resistance to applied pressure is the strongest near the wall of the capillary and becomes weaker the closer it is to the centre, the magnitude of this force is dependent on the radial distance from the centre of the capillary. The balance of force is expressed by the following equation\(^2\):

\[
\pi r^2 \Delta P = -2\pi rL_T \eta \frac{dv_m}{dr}
\]  
(Eq. 1.8)

where \(r\) is the radial distance from the centre of the capillary; \(\Delta P\), the pressure difference applied between the inlet and outlet; \(L_T\), the total length of the capillary; \(\eta\), solution viscosity; and \(v_m\), mobilization velocity. It can be rearranged to separate variables \(v_m\) and \(r\), and then be integrated to yield the following equation:

\[
v_m = \frac{\Delta P}{4L_T \eta} (r_c^2 - r^2)
\]  
(Eq. 1.9)

where \(r_c\) is the radius of the capillary. The velocity difference between the centre and the wall of the capillary can be reduced by minimizing the pressure difference; shortening the
length of the capillary; employing lower viscosity solution; and most importantly, using a narrow diameter capillary. In addition, strong electric field can be maintained during mobilization to reduce band broadening due to laminar flow profile\textsuperscript{33}.

1.2.4.2 \textit{Electrophoretic mobilization}

Capillary electrophoresis is a separation method that is uniquely unaffected by band broadening that plagues other pressure-driven chromatographic separations. This is due to flat flow profile achieved by wall-driven electroosmotic flow. Unfortunately for cIEF, EOF is usually suppressed and cannot provide adequate forward movement for the focused analytes. On the other hand, cIEF can take advantage of another migration mechanism that shares flat flow profile: electrophoretic migration, also known as chemical mobilization. It is accomplished as a result of the balance between electrostatic and drag force in solution:

\[
qE = 6\pi \eta r_e v_m \tag{Eq. 1.10}
\]

where \(q\) refers to the charge of the particle; \(E\), applied electric field; \(\eta\), viscosity; \(r_e\), effective radius of the particle; and \(v_m\), mobilization velocity. Electrophoretic mobility, \(\mu_{ep}\), is defined as the mobilization velocity per unit field. Rearranging (Eq. 1.10) produces an expression for electrophoretic mobility as the following, which describes proportionate dependency of mobilization velocity on the charge of the analyte:

\[
\mu_{ep} = \frac{v_m}{E} = \frac{q}{6\pi \eta r_e} \tag{Eq. 1.11}
\]

Analytes at the end of focusing are stationary at their isoelectric points, where the bulk of analyte population is neutral at their Gaussian maximum. To introduce charge to these neutral particles, catholyte at the outlet is replaced with an acidic or salt mobilizer.
solution. Since all solutions and capillary are under electric field, existing hydroxyl anions in the capillary continue to migrate toward anode while weak base anions in the mobilizer solution replace them (dotted chloride trace in Figure 1.5). Replacement of strong base with weak base or salt anions provide carrier ampholytes and analytes with partial positive charges, enabling their electrophoretic migration toward cathode. Influx of mobilizer ions as well as migration of CAs increase electric current in the capillary gradually. A sharp rise in current signifies elution process of CAs and analytes. Mobilizer anions are quenched by positively charged species inside capillary (inset in Figure 1.5), but are collected in anolyte after migrating through it as illustrated by the dotted chloride trace in Figure 1.5.

![Figure 1.5](image)

**Figure 1.5** Computer simulated concentrations, pH, and conductivity during chemical mobilization in a 3-cm capillary, boundaries of which are indicated by vertical dotted lines. The bottom trace of overlapping Gaussian peaks illustrate migration of carrier ampholytes while four sharp peaks labelled Hbs are hemoglobin isoforms (augmented by 100-fold and offset by 25 mM) show positions of the analytes. Anolyte of 100-mM H₃PO₄ and cathodic mobilizer of 40-mM NaOH and 20-mM HCl are represented by appropriately labelled solid and dotted lines. Another solid trace labelled pH illustrates the pH gradient while top trace labelled cond. shows conductivity. Inset: concentration of chloride in the space occupied by carrier ampholytes. Reprinted with permission from Thormann and Mosher’s work34. Copyright © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.
Simulated traces in Figure 1.5 describe a moment during chemical mobilization, where CAs and analytes have already partially exited out of the capillary into the catholyte. CAs exit and continue migrating into catholyte in isotachophoretic fashion with sodium cations being the leading electrolyte. Flattening of the pH gradient is also observed in catholyte, as mobility of carrier ampholytes increase in catholyte. Slight conductivity dips where protein peaks are focused demonstrate limited conductivity of proteins compared to carrier ampholytes. While cathodic mobilization described here is conventionally employed in cIEF, anodic mobilization can also be accomplished by replacing anolyte with a solution of weak acid or salt in a similar manner.

1.2.5 Detection

Mechanism of separation in capillary isoelectric focusing is fundamentally different from other common chromatographic separation techniques. Separation of analytes in chromatography is based on differential affinity towards the stationary phase; equilibrium of analyte partition between stationary and mobile phase is the key discriminating feature. For analytes to be separated, they must spend some time in the mobile phase to gain migration distance that distinguishes them from other analytes. On the other hand, separation in capillary isoelectric focusing is rather static: once analytes find their isoelectric points along the pH gradient, they must remain there to stay separated. This poses a unique challenge in terms of detection. Unless the entire column is detected as a whole like the example in Figure 1.4 above, the analytes must be mobilized towards the detector. In the following sections, various detection methods for cIEF are discussed, with greater emphasis on on-column optical detection and online mass spectrometry, which are particularly relevant to this thesis.
### 1.2.5.1 Whole column imaging

Whole column imaging is the fastest detection method available for capillary isoelectric focusing. Total analysis time with whole column imaging without counting preparatory rinsing steps is in the range of 5 minutes, as detection is near instantaneous\(^{35}\). There is a wide range of optical detectors to choose from, including refractive index gradient\(^{36}\), fluorescence\(^{37}\), and UV-visible absorbance detectors\(^{38}\). In addition to short analysis time, it allows real-time monitoring of the separation process and can be used to shed light on the mechanism of separation during cIEF\(^{28}\). Despite these advantages, its application is limited by its inability to pair with non-optical detectors, such as mass spectrometer, and by restricted choice of capillaries. As mechanical scanning with the detector often produces noise, a short capillary of a few centimetres in length is used. This is because short capillaries can be detected as a whole by a large enough linear charge coupled device (CCD)\(^{38}\). Using a short capillary is also necessary since whole column imaging requires the protective polyimide coating of the capillary to be removed, making it extremely fragile. Considering the high cost of internally coated capillaries, necessity of removing the external protective coating can become cost prohibitive. Moreover, short length of the capillary limits peak capacity, which, in turn, limits resolution. Whole column imaging is an excellent detection method for rapid analysis of moderate number of analytes and study of real-time molecule interaction during cIEF. Though it has a few limitations, it is an exceptional tool for several key applications.

### 1.2.5.2 Single-spot on-column optical detection

On-column detection with a small optical window is the method conventionally used in capillary electrophoresis (CE) and isoelectric focusing. It involves removing a small
length of external polyimide coating near the outlet of the capillary to preserve flexibility in the rest of the capillary. Both UV-visible\textsuperscript{39} and fluorescence\textsuperscript{40} absorbance detection are possible. With mobile separation like CE, this mode of detection is intuitive because analytes are already migrating during separation with electroosmotic flow (EOF) and their own electrophoretic mobility. Since many cIEF experiments are conducted using existing CE instrumentation, on-column absorbance detection is easily employed for cIEF as well. For UV-visible absorbance experiments, 280-nm incident light is usually chosen as commercial carrier ampholytes absorb lower wavelength light.

While applying this mode of detection to cIEF does not require any additional instrumentation, it necessitates mobilization step. Unfortunately, many mobilization methods, especially pressure mobilization, have an adverse effect on resolution. More importantly, additional mobilization step by several folds, as mobilization typically takes much longer than focusing. Despite these disadvantages, single-spot on-column optical detection is an easy choice that can be applied with an existing CE setup. It also does not require time-consuming optimization of the detector. These advantages make it especially suitable for optimization of experimental parameters\textsuperscript{41-43} and study of new coatings\textsuperscript{22, 44}, although it is also used for high-efficiency separation of complicated samples\textsuperscript{45-46}. Furthermore, it is often employed to track experimental progress when coupling with an additional external detector such as mass spectrometer.

1.2.5.3 Mass spectrometry

Advantages of employing mass spectrometer (MS) as a detector are numerous: it provides an additional dimension of separation by mass-to-charge ratio of analytes; it has
exceptional resolution and sensitivity; and it can be used to identify unknown proteins by comparing their fragmentation patterns with those in the established database. However, several experimental conditions of cIEF are incompatible with direct coupling with MS, such as inadequate volatility of solutions, incompatibility of anti-convective media and carrier ampholytes with ionization, and difficulty with incorporating ionization modules to allow online tandem coupling. To address these challenges, organic volatile solutions have replaced traditional inorganic acid and base solutions, as well as less-volatile polymeric separation media\textsuperscript{47-49}. Moreover, the presence of carrier ampholytes supress protein ionization and reduce their signal intensity\textsuperscript{47}. Efforts have been made to eliminate\textsuperscript{50-51}, reduce concentrations of\textsuperscript{50,52}, or replace carrier ampholytes with amino acids\textsuperscript{53-54}.

For online cIEF-MS, electrospray ionization (ESI) is most frequently used, coupled with a wide range of mass analyzers. Coupling with ESI is usually accomplished with a type of sheath liquid interface that serves to complete electric circuit while supplying with mobilizer solution. In addition, sheath liquid helps to relieve flow rate mismatch between eluent of narrow capillary and minimum requirement to maintain stable electrospray. Unfortunately, its unavoidable side effect is dilution of analytes by the sheath liquid.

Close behind ESI in popularity is matrix-assisted laser desorption/ionization (MALDI). Online coupling of MALDI is accomplished with automated fractionation and sheath-liquid-assisted sample deposition\textsuperscript{55-56}. Sheath liquid in MALDI also serves similar functions as in ESI: closing electric circuit and supplying with mobilizer solution. Ionization matrix is either pre-deposited on deposition targets or added with another capillary. A great advantage with MALDI is its leniency with carrier ampholytes that supress ionization
when compared to ESI\textsuperscript{57}. It has been demonstrated that samples containing up to 5\% CAs can be ionized as opposed to 1\% with ESI. Furthermore, offline coupling of MALDI allows analysis of complex samples through extraction and purification of separated fractions. Recently, Horká \textit{et al.} demonstrated identification of bacteria in complex plant samples using cIEF-MALDI\textsuperscript{58}. High ampholyte tolerance and opportunity for offline cleanup makes MALDI a great ionization method for complex samples and low-abundance analytes. However, it is more labour-intensive than ESI as it requires optimization of ionization matrix, and it is especially time-consuming if offline extraction is incorporated.

1.3 \textbf{Research Objectives}

1.3.1 \textbf{Reproducibility of capillary isoelectric focusing}

Successful capillary coating for application in cIEF must meet a few requirements: it must be stable over many runs; protect capillary wall from analyte adsorption; and suppress electroosmotic flow. Carrier ampholytes employed in cIEF must generate reproducible and linear pH gradient, in addition to accurately determining \textit{pl} of analytes. Before any coating or CAs can be used, they must be examined to determine how well they meet these criteria. Neutral coating from Beckman Coulter was available in both 50- and 30-\textmu m diameter, which was suitable for accomplishing research objectives in Chapter 3. Fluka\textsuperscript{®} CAs was found to produce repliable pH gradient at high resolution in our preliminary studies. To our knowledge, neither Beckman Coulter neutral coating nor Fluka\textsuperscript{®} CAs has been examined for reproducibility previously. In Chapter 2, reproducibility of cIEF with peptide \textit{pl} markers and myoglobin is evaluated to establish groundwork for Chapter 3. A 50-\textmu m capillary is used to allow empirical comparison with 30-\textmu m capillary.
1.3.2 Optimization for rapid capillary isoelectric focusing

In this era of proteomics and metabolomics, analysis of a multitude of complex samples has become the norm. Although cIEF is an exceptional tool for separation of biological samples, its long analysis time is hindering its widespread application. Its shortcomings are especially pronounced when compared to ultra-performance liquid chromatography that can profile multivariate metabolome within 10 minutes\textsuperscript{59} and targeted screening within 2 minutes\textsuperscript{60}. A narrow diameter capillary can shorten analysis time by reducing sample volume without sacrificing separation efficiency. It is also more lenient with band broadening caused by hydrodynamic mobilization. In Chapter 3, optimization for reduction in analysis time is achieved by adjusting sample composition, mobilization methods, and focusing time. Their effects on resolution and sensitivity are also described.
Chapter 2: REPRODUCIBILITY OF CAPILLARY ISELETRIC FOCUSING IN 50-μm INNER DIAMETER NEUTRAL CAPILLARY
2.1 Introduction

From very early on, students of science learn about factors that determine success of a scientific experiment. They are encouraged to comment on precision and accuracy of their experimental data and discuss sources of error, before they learn to interpret them. Such exercises prepare scientists to evaluate results of their experiments, which would become much more complicated than measuring the length of a stick multiple times. Evaluating the outcome of an experiment means something different for various disciplines of science. For an organic chemist, it may mean successful synthesis of a new compound with desired properties at a high yield. For a physical chemist, it may mean behaviours of a system agreeing closely with theoretical predictions. Regardless of the particular discipline, a common thread still runs through them: how precise, and how accurate? This is especially true for analytical chemists, and it is commonly referred to as reproducibility. Reproducibility is an important measure of analytical capability, along with sensitivity and resolution. It describes whether a technique can be relied upon to give accurate results with small variation. Before any technique can be applied to meaningful samples, its reproducibility must be ascertained lest the hard-acquired data become inconsequential.

For capillary isoelectric focusing (cIEF), reproducibility depends on stability of capillary coating and reliable formation of pH gradient by carrier ampholytes (CAs). Most lab-prepared and commercial capillary coatings are variations of polyacrylamide-based coating pioneered by Hjertên\textsuperscript{21} and cellulose-based coating applied by Shen and Smith\textsuperscript{23}. Both of these coating methods employ silanol-linkage that is prone to cleavage in highly basic solutions. Although much progress has been made, many coatings only last for several
dozens of runs and must be monitored for signs of deterioration. When it deteriorates, suppression of electroosmotic flow (EOF) is no longer uniform across the capillary and analytes may adsorb to the exposed charges on the wall. This results in non-linear migration of focused peaks, and adversely affects both precision and pI accuracy.

Carrier ampholytes are complex co-polymerized concoctions of oligo-amines with α-β-unsaturated carboxylic acid that vary greatly in molecular weights. The proprietary synthetic method is rather chaotic, and linearity and range of pH gradient can vary from batch to batch. Since determination of pI assumes linearity of pH gradient, accuracy of calculated pI depends on the particular CAs employed in the analysis.

Before optimization for short analysis time can be conducted, reproducibility of coating and consistency of the compositions of CAs should be established. There are only a handful of publications using Beckman Coulter neutral coating and Fluka® carrier ampholytes, none of which discusses reproducibility. In this chapter, stability of neutral coating in 50-μm capillary is studied to determine its longevity. Accuracy of pI deduction is established by calculating pI values of myoglobin isoforms with help of peptide pI standards. Results of replicate cIEF runs will provide insight into effect of capillary conditioning and analyte adsorption.

2.2 EXPERIMENTAL

2.2.1 Instrumentation

All cIEF experiments were performed on MDQ Capillary Electrophoresis System by Beckman Coulter (Brea, CA, USA) using an ultraviolet (UV) absorbance detector equipped with a 280-nm filter. A standard capillary cartridge compatible with Beckman Coulter CE
systems was used with a 100×200 μm aperture. All experiments were performed on a single 50-μm ID, 350-μm OD, 50-cm $L_T$ neutral coated capillary (Beckman Coulter) with a premade UV window at 40 cm from the inlet. The capillary was kept at 20°C while the sample compartment was kept at 15°C.

2.2.2 Chemicals and materials

Ammonium hydroxide (certified ACS), glacial acetic acid (certified ACS), 85% phosphoric acid (certified ACS), $N,N$-dimethylformamide (certified ACS), and methanol (HPLC grade) were purchased from Fisher Scientific (Nepean, ON, Canada). Arginine (TLC, ≥98%), iminodiacetic acid, myoglobin (from horse heart), sodium hydroxide (95–100%), and urea (for electrophoresis) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Peptide standards ($pI$ 10.0, 9.5, 7.0, 5.5, and 4.1), cIEF gel polymer solution, and carrier ampholytes (Fluka® brand, pH 3.0–10.0) were obtained from Beckman Coulter Inc. (Brea, CA, USA). All aqueous solutions were prepared in deionized water.

2.2.3 Preparation of $pI$ standards

Master $pI$ standards mixture was prepared in 50% v/v cIEF gel in water with 3 M urea. The master mixture consisted of 2% w/v CAs, 30 mM arginine (ARG), 1.6 mM iminodiacetic acid (IDA), and 0.8% v/v of each peptide $pI$ standard. It was kept at 4 °C for the duration of reproducibility experiments. To prepare the sample solution, 2 μL of 1% w/v myoglobin solution in water was mixed with 48 μL of the master mixture.

2.2.4 Methods

At the beginning of each set of 10 cIEF runs, EOF was measured and capillary was conditioned. EOF was measured with 0.05% v/v $N,N$-dimethylformamide (DMF) as a
neutral marker in 50 mM phosphate buffer at pH 7 (PB7). A Quick EOF measurement method was adapted from Williams and Vigh. First, the neutral capillary was rinsed with water for 2 min and PB7 for 5 min at 35.0 psi. DMF solution was then injected for 3.5 s at 2.0 psi. The DMF plug was pushed with 2.0 psi for 1 min, followed by another cycle of injection and forward pressure. With both ends of the capillary immersed in PB7, +15 kV was applied to the outlet of the capillary for 5 min. Finally, after another injection of DMF, a forward pressure of 2.0 psi was applied for 5 min with the UV detector set to 200 nm. After each set of 10 runs, the capillary was conditioned by rinsing with water for 5 min, methanol for 2 min, and separation medium (50% v/v cIEF gel in water) for 5 min at 50.0 psi.

At the start of each run, the capillary was rinsed with 4.3 M urea for 3 min and with water for 2 min at 50.0 psi. Sample solution was then injected at 25 psi for 99.9 s. Focusing of CAs and pI standards was accomplished by applying +25 kV to the inlet of the capillary while the inlet and the outlet were submerged in anolyte (200 mM H₃PO₄) and catholyte (300 mM NaOH), respectively. The extent of focusing was monitored by capillary current: as the CAs and pI standards focus near their isoelectric points, the ion movement in the solution is drastically reduced thereby reaching a low plateau in electric current. The separation current was deemed to have plateaued at 15 min into focusing, at which point mobilization was initiated by submerging the outlet of the capillary in mobilizer solution (350 mM acetic acid) while +30 kV was applied to the inlet. At the end of the run, the capillary was rinsed with water for 5 min at 50.0 psi. In order to minimize electrolyte contamination, electrolyte, mobilizer, and sample solutions were replaced every 5 runs. At the end of the day, the capillary was rinsed with water for 2 min then with separation medium for 10 min at 50 psi. The capillary was stored with both ends submerged in water.
2.3 RESULTS AND DISCUSSION

2.3.1 Peak identity and determination of myoglobin pl

Identity of each pl standard peak was confirmed by comparing with a replicate cIEF experiment containing the corresponding pl standard. Myoglobin isoforms were identified in a similar manner and confirmed by comparing their calculated pl values with literature. Their pl values were calculated using two pl markers at 5.5 and 7.0 for Myo I and those at 7.0 and 9.5 for Myo II as standards. It was assumed that the pH gradient was linear with migration time within the short range between the two flanking pl standards. A representative electropherogram with labeled peaks is shown in Figure 2.1.

![Electropherogram](image)

Figure 2.1 A representative electropherogram of a complete cIEF experiment using pl 3-10 carrier ampholytes and 5 peptide pl standards at pl 10.0, 9.5, 7.0, 5.5, and 4.1. Two myoglobin isoforms were observed as labelled in the inset. Inset: magnified electropherogram from 40 to 45 minutes.
2.3.2 Reproducibility

Run-to-run reproducibility and coating stability are important parameters to study before embarking on a multivariate optimization in cIEF. To minimize variation caused by sample preparation errors, all sample solutions were aliquoted from the same master mixture. All electrolyte solutions were also drawn from their respective stock solutions. Migration times of the standard pI markers and calculated pI values of the two myoglobin (Myo) isoforms over 60 replicate runs are found in Figure 2.2. Partial electropherograms of 10 replicate runs from the 21st to 31st are compiled in Figure 2.3 to help visualize change in migration times.

Figure 2.2 Migration times of standard pI markers (dotted lines) and calculated pI values of two myoglobin isoforms (solid lines) over 60 replicate runs. Fresh sample and electrolyte solutions were used every 5 runs and the capillary was conditioned every 10 runs. Disconnects on pI 4.1 and pI 10.0 traces are due to analyte peaks being outside the pre-set analysis time window.
Figure 2.3 Partial electropherograms approximately from pH 7.5 to 4.5, showing myoglobin isoforms and pI standards 7.0 and 5.5. The order of each experiment among the 60 replicate runs in Figure 2.2 is indicated on the left side.

Migration times of all five pI standards display similar patterns: they increase in subsequent runs until the capillary is conditioned, at which point migration times decrease substantially. This trend is illustrated in Figure 2.3 in which migration times increase from the 21st to the 30th run with a significant jump after the 25th run. The capillary was conditioned after the 30th run, and a drop in migration time is observed between the 30th and the 31st run. Conditioning after the 30th run reduces migration times of the analytes to be similar to those of the 21st run, which was also right after a conditioning. These trends lead to a few important conclusions: (1) there is variation in sample solutions despite their common source; (2) such variation does not greatly affect accuracy and precision calculated pI values of proteins; (3) after every run, some residual components reduce EOF;
(4) capillary conditioning reverts the effect of these residual components; and (5) the integrity of the coating is maintained for at least 50 runs.

2.3.2.1 Variation in sample solutions

Sample and electrolyte solutions were changed every 5 runs to minimize reduction of electrolyte conductivity due to isotachophoretic loss of sample components into the electrolytes\textsuperscript{42}. If the extent of electrolyte dilution were still significant, there would be two observable consequences: a gradual decrease in mobilization time caused by reduced electrolyte conductivity and reversion of such effect when fresh electrolytes are used. Neither of these effects was consistently observed over 60 cIEF runs, thus it can be concluded that electrolyte dilution by isotachophoretic drift is negligible if fresh solutions were used every 5 runs.

On the other hand, a sudden and significant change in migration time, where it is greater than the standard deviation of the previous 5 runs, is observed between the 5\textsuperscript{th} and 6\textsuperscript{th}, 15\textsuperscript{th} and 16\textsuperscript{th}, and 25\textsuperscript{th} and 26\textsuperscript{th} runs. As these jumps in migration time are not consistent with predicted effects of electrolyte dilution, they must be caused by the variation in sample mixture compositions. CAs are traditionally synthesized by copolymerization of isomeric amines and peptides which produces a highly complex mixture of diastereomers varying slightly in their pK\textsubscript{a} values\textsuperscript{64}. While heterogeneity of CAs plays a crucial role in creating a smooth pH gradient, it can also result in inconsistent ampholyte composition between aliquots. Furthermore, when the total volume of CAs used is extremely small, as in the case of cIEF, the variation in compositions even in the same vial can be amplified. Mass distribution and pK\textsubscript{a} range of several commercial CAs have
already been thoroughly studied by Righetti and others using Rotofor fractionation and CE-MS^{65-69}. It may provide further support for this heterogeneity hypothesis to study the reproducibility of CA composition following similar analytical methods as Righetti.

2.3.2.2 Effect of CA variation on calculated pI values

In contrast to large variation in migration time, calculated pI values of two myoglobin isoforms, Myo I and Myo II (red trace in Figure 2.2), remained relatively constant. Relative standard deviations (RSD) of calculated pI were 0.11\% and 0.27\%, respectively. Their relative standard deviations are less than a tenth of the smallest relative standard deviation of migration time, which is 4.9\% for the pI 10 marker. This demonstrates that cIEF can reliably determine pI values of proteins, despite the marked variation in migration time.

Unfortunately, accuracy of calculated pI is not on par with the exceptional precision. Their calculated pI values were 6.71±0.01 and 7.07±0.02, which are both lower than published values by Sigma-Aldrich: 6.8 and 7.2. Isoelectric points in cIEF are difficult to measure accurately as they are affected by temperature and salt concentration in the medium^{62}. Regrettably, presence of salt is rather unavoidable when employing electrophoretic mobilization. As a result, pH gradient is not completely linear, especially in basic and neutral pH regions that commonly have poorer ampholytes^{25}. There are hidden regions of non-linearity in the gaps between pI markers, although pH gradient appears to be linear with the few markers employed^{70}. Using two pI markers that bracket analyte peaks instead of all five allows more accurate calculation of pI by avoiding non-linear areas along the pH gradient. Wu and Huang tested more than 200 proteins by using this method,
and found that they were able to determine their pl within 0.1 of the literature values\textsuperscript{71}.

Calculated pl values of myoglobin isoforms in this chapter corroborate the results of their study, and are accurate representations of isoelectric points given the limitations.

2.3.2.3 \textit{Calculation of EOF and its effect on migration times}

At the end of each set of 10 runs before the capillary was conditioned, EOF was measured. The calculated EOF values are presented in Table 2.1.

<table>
<thead>
<tr>
<th>Run, n</th>
<th>$\mu_{eo}$ ($10^{-5}$ cm$^2$ V$^{-1}$ s$^{-1}$)</th>
<th>$t_n$ (min)</th>
<th>$t_{n+9}$ (min)</th>
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<td>1</td>
<td>2.02±0.03</td>
<td>42.075</td>
<td>44.554</td>
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<tr>
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<td>44.058</td>
<td>50.513</td>
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<td>44.058</td>
<td>49.500</td>
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<td>45.367</td>
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<td>41</td>
<td>-</td>
<td>42.433</td>
<td>46.413</td>
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<tr>
<td>51</td>
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<td>36.679</td>
<td>39.292</td>
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<tr>
<td>61</td>
<td>1.85±0.03</td>
<td>-</td>
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</tbody>
</table>

Table 2.1 The average electroosmotic mobility of three replicate measurements and migration times of the pl 7 marker. Electroosmotic mobility ($\mu_{eo}$) was measured at the start of each set of 10 runs, before run $n$ for the row. Migration time, $t_n$, refers to that of the run number indicated by the leftmost column while $t_{n+9}$ refers to the run that is 9 runs after the run number, $n$.

Calculation of EOF, $\mu_{eo}$, follows the method of Williams and Vigh\textsuperscript{63} using the formula below:

$$\mu_{eo} = \frac{\left[ (t_{N3} - t_{N2}) - (t_{N2} - t_{N1}) \right] v_m L_T}{v_{prog} \left( t_m - \frac{t_{\text{ramp-up}}}{2} - \frac{t_{\text{ramp-down}}}{2} \right)}$$  (Eq. 2.1)

where $t_{N1}$, $t_{N2}$, and $t_{N3}$ are migration times of each neutral marker in the order of appearance in the electropherogram, $v_m$ is the pressure mobilization velocity, $L_T$ is the total length of the capillary, $v_{prog}$ is the applied potential, $t_m$ is the electrophoretic migration time with applied potential, and $t_{\text{ramp-up}}$ and $t_{\text{ramp-down}}$ are the times it takes to linearly change the

*Measurement of electroosmotic mobility was unsuccessful due to an error in the instrument during an automated sequence.
applied potential from 0 to $V_{prog}$, and vice versa. Ramp times were measured using Williams and Vigh’s method$^{72}$.

Throughout the experiment, EOF stayed in the low $10^{-5}$ cm$^2$ V$^{-1}$ s$^{-1}$, which is considered sufficiently slow for cIEF applications. Although EOF in this range is almost negligible, it follows logically that faster EOF would lead to slightly shorter migration time of the $pI$ markers. Even with very slow forward EOF, proteins would incrementally move towards the detector during focusing, resulting in slightly shorter migration time. Data in Table 2.1 demonstrate this correlation, although it is not exactly a linear proportionality. Gradual increase of migration time in the course of next 9 runs is evident in both Figure 2.2 and Table 2.1, although magnitude of increase varies. This can be attributed to analytes getting retained by residual proteins and peptides adsorbed to the capillary wall$^{43,73}$. For the first 30 runs, this increase is reverted by capillary conditioning as shown by consistent migration times ($t_n$ column in Table 2.1). Unfortunately, after the 40th run, the reversion is accompanied by further reduction in migration time. This is likely due to the sudden increase in electroosmotic mobility, which may indicate deterioration of coating. The reduction in migration time becomes more severe after capillary conditioning following the 50th run. Deterioration of coating is further corroborated by appearance of baseline noise after 50 runs. As extended rinsing is known to be detrimental to some capillary coatings$^{22}$, conditioning after every 10 runs likely plays an important role in coating’s demise. However, migration times will continue to increase without capillary conditioning, and greatly extend analysis time. To keep analysis time to a minimum, regular conditioning is essential.
Stability of the capillary coating has always been an important parameter of a successful coating method. Integrity of the neutral coating starts to decline around 40 runs, as demonstrated by reduction in migration times. However, quality of analysis is only affected after 50 runs, when relatively large fluctuation in the calculated pI values of the myoglobin isoforms and appearance of random noisy peaks in the electropherogram are observed. The cIEF gel medium is a viscous polymer solution that can suppress EOF, similar to glycerol-water medium in Bunsel and colleagues' work\textsuperscript{74}. Hence deterioration of coating is somewhat masked by EOF suppression effect of the medium. Despite continued suppression of EOF, it is recommended to replace capillary after 50 runs.

2.4 Conclusions

The verdict on precision and accuracy of cIEF using Beckman Coulter neutral coating and Fluka\textregistered ampholytes is unfortunately not a concise one-word answer. Precision of pH gradient formation by carrier ampholytes was rather poor due to analyte adsorption and heterogeneity of ampholytes themselves. Relative standard deviations of migration times varied from 4.9\% to 8.0\% over 60 runs. Fortunately, calculated pI for myoglobin isoforms were much more precise, at 0.11\% and 0.27\% RSD for Myo I and Myo II, respectively. Accuracy of pI determination was on par with literature, with $\Delta pI$ of 0.1 for both isoforms. The discrepancy between experimental and published pI stems from non-linearity of pH gradient due to presence of salt in separation medium. With these caveats and limitations, the verdict on cIEF as a technique for precise and accurate separation and identification of proteins would be "suitable for analysis".
In addition to establishing reproducibility of cIEF, some insights into several experimental details came to light. The neutral capillary was “suitable for analysis” for 50 consecutive runs, after which quality of analysis began to be affected. Signs of deterioration actually emerged after 40 runs in the form of increased electroosmotic mobility, but determination of myoglobin pI remained unaffected for another 10 runs. Capillary conditioning after every 10 runs was the main culprit for the coating’s demise, although basic catholyte and ampholytes also played a role. Unfortunately, capillary conditioning is a necessary evil as between-run rinsing alone was not sufficient to remove residual analytes. With findings of this chapter, we can now proceed to the next phase of our quest for rapid cIEF analysis, equipped with a better understanding of the limitations and capabilities of the technique.
Chapter 3: OPTIMIZATION FOR RAPID CAPILLARY ISOELECTRIC FOCUSING WITH 30-μM INNER DIAMETER NEUTRAL CAPILLARY
3.1 Introduction

When Hjertén and Zhu introduced capillary isoelectric focusing (cIEF) in 1985\textsuperscript{20}, it seemed like it would be the next analytical standard for protein separation and identification. It possessed many characteristics of a successful bioanalytical technique: sensitive, reproducible, automatable, with high resolution and minimal sample consumption. Considering that two-dimensional gel electrophoresis (2DGE) with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) formed the basis of proteomics in the preceding decades, their high-resolution counterparts, mass spectrometry (MS) and cIEF, appeared to be the perfect replacements for the new era of modern proteomics. So it is surprising to find almost three decades later the ubiquitous presence of MS in all facets of protein analysis without equally widespread application of cIEF.

The rise of MS in proteomics began early when individual gel spots from 2DGE were analyzed offline by MS for accurate mass and structural identification of protein fragments\textsuperscript{75}. However, limitations of 2DGE as a high-resolution separation technique soon became apparent. Such limitations include: underexpression of proteins at the extreme ends of the separation parameter, \textit{e.g.}, molecular size or pI; difficulty in detecting low abundance proteins without prior enrichment\textsuperscript{76}; laborious gel preparations and protein spot extractions; and long total analysis times. Replacing 2DGE with cIEF can address many of these challenges, as cIEF is capable of rapid, sensitive, reproducible, and high-resolution separation\textsuperscript{77}. Furthermore, if cIEF and MS are coupled for two-dimensional separation, it is possible take advantage of the massive library of MS analysed proteins. However, online
interfacing with MS requires considerable revamping of the existing on-column detector setup due to flow rate mismatch and solution incompatibilities. Recent successful strategies for online cIEF-ESI-MS coupling include flow rate adjustment with sheath liquid\textsuperscript{48,53}, reduction or replacement of commercial ampholytes\textsuperscript{50,52}, pre-MS trap of MS-incompatible components\textsuperscript{78-79}, and elimination of polymeric separation media\textsuperscript{49,80}.

In addition to online coupling with MS, cIEF itself can be optimized to become more suitable for current applications. In this era of genomics, proteomics, and metabolomics, the complete profile of cellular biomolecules has become the standard unit of individual analysis. For applications such as biomarker screening or drug discovery, thousands of such profiles must be generated within a short period of time. To meet the demands of high-throughput screening, analysis time of cIEF must be reduced to a fraction of the current 30-min range. While there are many general experimental parameters that can be adjusted for minimum analysis time, a careful theoretical evaluation of the separation mechanism can lead to a more systematic approach.

Successful cIEF analysis, like any other separation technique, has two requirements: separation of the analytes and their detection. Mechanism of separation in cIEF is very unique, because separation gradient remains static; analytes find their isoelectric points (pI) along the established pH gradient and remain there. For successful detection of these analytes, they must then be mobilized toward the detector of choice without interrupting the pH gradient. Therefore, optimization of cIEF for minimum analysis involves two distinct components: shortening focusing time and speeding up mobilization. One of the most effective ways to shorten focusing time is to reduce capillary volume, although this is
at a direct cost of analyte sensitivity. Establishing pH gradient with carrier ampholytes (CAs) happens proportionally faster if there are fewer ampholyte ions. Reducing capillary volume can be accomplished by using a shorter or narrower capillary. Another way to shorten focusing time is to reduce the viscosity of the separation medium by adjusting the concentration of CAs or anti-convective additive. Lowered viscosity enables swifter movement of ampholyte and analyte ions to their respective pl, although it may adversely affect separation efficiency by tolerating diffusion.

After focusing is complete, mobilization is often accomplished by electrophoretic methods to minimize peak broadening resulting from pressure-driven flow\textsuperscript{81}. This phenomenon was experimentally observed by Mack and colleagues, who demonstrated resolution of two synthetic peptides using chemical mobilization\textsuperscript{42}; these two peptides previously co-migrated when using pressure migration. Unfortunately, ways of accelerating chemical mobilization are limited, as chemical mobilization requires electrophoretic introduction of mobilizer cations into the capillary and slow titration of ampholyte and analyte molecules. However, since mobilization is typically the longer half of the focusing-mobilization pair, reducing mobilization time is crucial in achieving fast cIEF. Our approach to this dilemma is to introduce pressure mobilization while taking measures to maintain separation efficiency. Recall (Eq. 1.9), which describes laminar profile in pressure-driven flow:

\[
v_m = \frac{\Delta P}{4L \eta} \left( r_c^2 - r^2 \right) \quad \text{(Eq. 1.9)}
\]
The most efficient way to minimize velocity difference between the centre and the wall of the capillary is to reduce the capillary diameter. It can also be adjusted by narrowing the pressure difference, shortening the length of the capillary, and employing lower viscosity separation medium.

To introduce pressure mobilization with minimum compromise to separation efficiency, a 30-μm neutral capillary, which is much narrower than the typical 50- or 100-μm capillaries, is examined. Experimental parameters, such as sample solution composition and length of focusing are optimized with a careful consideration on resolution and sensitivity. Various mobilization methods are also tested to achieve the fastest analysis with minimal loss of resolution.

### 3.2 Experimental

#### 3.2.1 Instrumentation

All cIEF experiments were performed on MDQ Capillary Electrophoresis System from Beckman Coulter (Brea, CA, USA) with an ultraviolet (UV) absorbance detector equipped with a 280-nm filter. A standard capillary cartridge compatible with Beckman Coulter CE systems was used with a 100×200 μm aperture. A 30-μm ID, 350-μm OD, 50-cm LT neutral coating capillary (Beckman Coulter) with a premade UV window at 40 cm from the inlet was used. The capillary was kept at 20°C or 25°C while the sample compartment was kept at 15°C.

#### 3.2.2 Chemicals and materials

Ammonium hydroxide (certified ACS), glacial acetic acid (certified ACS), 85% phosphoric acid (certified ACS), and methanol (HPLC grade) were purchased from Fisher
Scientific (Nepean, ON, Canada). Arginine (TLC, ≥98%), iminodiacetic acid, glycerol (for electrophoresis, ≥99%), sodium hydroxide (95–100%), and urea (for electrophoresis) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Peptide standards (pI 10.0, 9.5, 7.0, 5.5, and 4.1), cIEF gel polymer solution, and carrier ampholytes (Fluka® brand, pH 3.0–10.0) were obtained from Beckman Coulter Inc. (Brea, CA, USA). All aqueous solutions were prepared in deionized water.

### 3.2.3 Preparation of pI standards

Sample solutions were prepared in either 50% v/v cIEF gel in water or 0–30% v/v glycerol in water. They were prepared fresh at the start of each day from aliquots of pI standards and carrier ampholytes (CAs) stored at 4°C, and solutions of 500 mM ARG and 200 mM IDA stored at room temperature. Each sample mixture contained 0.64% w/v CAs, varying concentrations of ARG, 1.6 mM IDA, and 0.8% v/v of each peptide standard.

For glycerol concentration experiments, two sample solutions were prepared: one solution was in 30% v/v glycerol in water while the other was simply in water. To create a sample solution with glycerol concentration between 0 and 30%, the two sample solutions were mixed in appropriate proportions. For experiments in which sample composition remained constant, the same sample solution was used to minimize variance resulting from sample preparation errors.

### 3.2.4 Methods

At the beginning of each run, the capillary was rinsed with 4.3 M urea for 2 min and with water for 3 min at 50.0 psi. The sample solution was then injected at 50.0 psi for 2 min. Focusing of the CAs and pI standards was accomplished by applying +30 kV to the
inlet of the capillary while the inlet and the outlet were submerged in anolyte (200 mM H$_3$PO$_4$) and catholyte (300 mM NaOH) solutions, respectively. Depending on the composition of the sample solution, mobilization was programmed to start 7–20 min after the start of focusing.

At the end of focusing, mobilization of the focused pI standards towards the detector was accomplished in one of three ways: chemical, pressure, or pressure-assisted chemical mobilization. During chemical mobilization (CM), the inlet and the outlet of the capillary were submerged in anolyte and mobilizer solutions, respectively, while +30 kV was applied to the inlet. During pressure mobilization (PM), a pressure of 1 psi was applied to the inlet of the capillary while the capillary remained under focusing conditions. During pressure-assisted chemical mobilization (PCM), the capillary was placed under chemical mobilization conditions while a pressure of 1 psi was applied to the inlet of the capillary.

At the end of each run, the capillary was rinsed with water at 50 psi for 5 min. At the end of the day, the capillary was rinsed with water for 2 min then with separation medium for 10 min at 50 psi. The capillary was then stored with both ends submerged in water.

### 3.3 Results and Discussion

#### 3.3.1 Arginine as a cathodic spacer

As Mosher and Thornmann clearly demonstrated in 1990, anodic and cathodic drifts can cause loss of analytes near the CAs pI termini. The mechanism of these drifts is isotachophoresis (ITP), in which catholyte and anolyte act as leading electrolytes for cations and anions, respectively. In cIEF-UV experiments, anolyte and catholyte solutions of high conductivity are commonly used to decrease local field strength, thereby reducing
mobilities of the electrolytes. Using solutions of high ionic concentrations has an additional benefit of increasing run-to-run reproducibility as they are resistant to dilution through accumulation of analyte species in the solution. However, electrolytes of high conductivity can cause temporary compression of the pH gradient due to diffusion of the electrolyte ions into the focusing space. To preserve linear pH gradient in the pH range of interest while protecting analytes from residual anodic or cathodic drifts, sacrificial ampholytes can be added to the sample mixture. Following the procedure published by Mack et al., IDA and ARG were added to the sample mixture to serve as anodic and cathodic sacrificial ampholytes. All five peptide pI standards (pI 4.1 to 10.0) were detected successfully with relatively linear pH gradient.

Arginine serves one additional function in cIEF-UV experiments: it is used as a cathodic spacer to ensure that analytes are focused on the inlet side of the detection window. As the detector window is placed 10 cm away from the outlet of the capillary, only 80% of the capillary length in a 50 cm capillary is viable separation space. The percentage of arginine should be optimized to focus all pI standards on the inlet side of the detector with the first peak being as close to the window as possible to ensure fast analysis. In a similar cIEF-UV experiment with a 50-μm neutral capillary, 8% v/v arginine in the sample mixture was found to be optimal (data not shown). However, when the same sample composition was used in a 30-μm neutral capillary, it took approximately 23 minutes after start of mobilization for the first peak to appear (Figure 3.1).
Figure 3.1 Electropherograms of three peptide pI markers (pI 10.0, 9.5, and 7.0) with varying concentrations of arginine in the sample mixtures. The sample mixtures were composed of 50% v/v cIEF gel. Mobilization started at 15 minutes. A: 0% (offset by 0.03 Au); B: 1% (offset by 0.02 Au); C: 3% (offset by 0.01 Au); and D: 8% v/v of 500 mM arginine.

To expedite mobilization, ARG composition was gradually reduced from 8% v/v to 3, 1, then 0% v/v as shown in Figure 3.1. Surprisingly, all pI markers were focused on the inlet side of the window even with no ARG in the solution (top trace). Theoretically, both basic peaks (pI 10.0 and 9.5) should be focused on the outlet side of the window because they are in the top 20% of the CAs pI range (pI 3–10). However, this prediction makes two important assumptions: (1) the pI range is indeed 3–10 as published; and (2) CAs pI range is spread linearly from the inlet to the outlet of the capillary.

The first assumption is easily refuted by a series of studies by Righetti et al., in which they analyzed four major brands of CAs\textsuperscript{25,65-67}. They have reported inconsistent pI
ranges, large proportions of poor ampholytes in the basic pH region, and nonlinear pH gradients near the ends of the pI range. Bio-Lyte exhibited basic pI range that is two units higher than their published values while other three brands had extended acidic ranges. In all four brands, up to 70% of the basic ampholytes were poor buffers, leaving only a small percentage of CAs to maintain the pH gradient. Steepening or flattening of the pI gradient near the ends of the pI range was also observed, which can alter the perceived pI range. Though the CAs used in the experiment are not one of the four brands studied by Righetti and others, it is reasonable to expect the published pI range to be a guideline rather than a precise parameter, given the inconsistencies found in the other four brands of CAs.

While the imprecise pI range may explain the large cathodic spacing to a certain extent, there are still a few concerns. First, it is difficult for this theory to solely account for the loss of 20% of the separation space, from 50 cm (total length of the capillary) to 40 cm (inlet to detector): the basic pI region would have to be extended by 2 units without extension of the acidic region. Such a large deviation was only observed with one segment of one of the four brands of CAs. A more important concern, however, is the discrepancies between 50- and 30-μm capillaries. If the pI range is solely responsible for the cathodic spacing, i.e., the pI range extends further into the basic region than published, then the same trends should be observed regardless of the capillary internal diameter. Therefore, the explanation for the large cathodic spacing should incorporate the impact of capillary internal diameter.

In Mosher and Thormann’s study of isotachophoretic drifts in isoelectric focusing (IEF), compression of the pH gradient in the basic region was observed when a highly
conductive buffer was used as catholyte. This resulted in temporary steepening of the pH gradient, producing a long region high pH composed of ampholytes and catholyte at the cathodic end of the IEF gel. This effect is especially pronounced when there is a large difference between the separation space and electrolyte reservoir volume. Thus, the large cathodic spacing observed in this experiment may be due to the steepening of the pH gradient caused by a high concentration catholyte, in addition to the imprecise pH range. The difference between 50- and 30-μm capillaries can be explained by the substantially smaller volume of the 30-μm capillary while the reservoir volume remained the same.

Since all pH standards can be detected without ARG in the sample mixture, no ARG was added to subsequent experiments. This would ensure fastest analysis possible without any observable adverse effects on separation quality.

### 3.3.2 Mobilization

The goal of this chapter is to seek ways of reducing analysis time without significant loss of analysis quality including resolution and sensitivity. The most convenient, although somewhat crude, method of reducing analysis time is to apply forward pressure. Three mobilization methods were tested: chemical, pressure, and pressure-assisted chemical mobilization. Peak width and peak height were monitored to serve as an indicators of resolution and sensitivity. Representative electropherograms from three modes of mobilization are shown in Figure 3.2 and results are summarized in Table 3.1 below. Resolution was calculated using the preceding peak as a reference with the formula:

\[
R_s = \frac{t_n - t_{n-1}}{\frac{1}{2} (w_n + w_{n-1})}
\]  

(Eq. 3.1)
where $t_n$ and $t_{n-1}$ are migration times of a peak and its preceding peak, respectively, and $w_n$ and $w_{n-1}$ are their widths.

![Figure 3.2 Electropherograms of four peptide $pI$ standards ($pI$ 10.0, 7.0, 5.5, 4.1) with three different mobilization methods. The sample mixture contained 50% v/v cIEF gel with no ARG. Mobilization started at 15 minutes. From bottom: chemical mobilization, pressure mobilization (offset by 0.01 Au), and pressure-assisted chemical mobilization (offset by 0.02 Au). The pressure applied at the inlet for pressure and pressure-assisted chemical mobilization was 1 psi.]

In terms of analysis time, pressure-assisted chemical mobilization (PCM) was the shortest, followed by pressure mobilization (PM), then chemical mobilization (CM). While comparison of analysis time between PM and CM is not meaningful as it depends on the magnitude of the pressure applied, a combination of both, PCM, should be faster than either mobilization mode; this effect was indeed observed. When comparing PCM to either CM or PM, reduction in elution time of each peak was proportional to its migration time. In other words, reduction in elution time was greater if a peak stayed in the capillary longer, e.g. $pI$
4.1 marker. This signifies linear rate of elution in all three mobilization modes, which implies pH gradient linearity as well as elution linearity. Linear rate of elution is also collaborated with linearity of pI versus elution time of each mobilization mode. The correlation coefficients of CM, PM, and PCM were found to be 0.989, 0.999, and 0.991, respectively.

Table 3.1 Peak heights and resolution of the peptide pI standards with three modes of mobilization: chemical, pressure, and pressure-assisted chemical mobilizations.

<table>
<thead>
<tr>
<th>Peak pI</th>
<th>CM Peak Height</th>
<th>Resolution</th>
<th>PM Peak Height</th>
<th>Resolution</th>
<th>PCM Peak Height</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0</td>
<td>32681</td>
<td>-</td>
<td>23409</td>
<td>-</td>
<td>18500</td>
<td>-</td>
</tr>
<tr>
<td>7.0</td>
<td>20972</td>
<td>63</td>
<td>21144</td>
<td>64</td>
<td>28726</td>
<td>65</td>
</tr>
<tr>
<td>5.5</td>
<td>37199</td>
<td>30</td>
<td>26093</td>
<td>21</td>
<td>32192</td>
<td>19</td>
</tr>
<tr>
<td>4.1</td>
<td>14816</td>
<td>25</td>
<td>21266</td>
<td>23</td>
<td>18085</td>
<td>23</td>
</tr>
</tbody>
</table>

Advocates of CM often quote loss of resolution due to band broadening caused by the laminar flow profile of pressure-induced mobilizations. The relationship between plate height and its contributing factors during pressure-induced mobilization can be described by the following equation:

$$H = \frac{r_c^2 + 12K r_c + 44K^2}{24D} \frac{r_c}{r_c + 2K} v_m + \frac{4K}{(r_c + 2K)k_d} v_m + \frac{2D}{v_m}$$  \hspace{1cm} (Eq. 3.2)

where $H$ is the plate height, $r_c$ is capillary radius, $K$ is the distribution coefficient of the analyte, $D$ is the diffusion coefficient, $v_m$ is the mobilization velocity, and $k_d$ is the rate constant of desorption. The first two terms on the right hand side of (Eq. 3.2) are contributions of protein adsorption and radial diffusion, whereas the last term accounts for effects of longitudinal diffusion. Assuming minimal adsorption in coated capillaries, (Eq. 3.2) can be reduced to the following as $K$ goes to zero:
During CM, effects of radial diffusion are minimized because wall-driven electrophoretic flow generates a flat flow profile. Thus, the theoretical plate height during CM only needs to account for longitudinal diffusion, and will be much smaller than the plate height during PM:

\[
H = \frac{r_c^2}{24D} v_m + \frac{2D}{v_m} \quad \text{(Eq. 3.3)}
\]

Typically, plate height during CM will be several hundredths of that during PM, as the magnitude of the second term is only a fraction of 1% of the first. However, by switching to a narrower diameter capillary, the first term can be somewhat reduced during PM. Since the radius of the capillary, \(r_c\), affects plate height to the second power, reducing the capillary diameter to a tenth reduces peak height to a hundredth. In the case of \(50-\mu\text{m}\) to \(30-\mu\text{m}\) capillary, reduction to a quarter is expected.

On the other hand, (Eq. 3.2) does not consider the effect of focusing voltage often applied during PM. A significant improvement in resolution when maintaining focusing conditions during PM is well known\(^{20,33}\). This effect is likely due to self-focusing properties of cIEF, bringing diffused protein peaks back to their \(p\)Is. Minárik and others observed almost a ten-fold reduction in the coefficient of the first term in (Eq. 3.3) when they increased the focusing voltage from 0 to 5 kV\(^{33}\). Considering that they were using a much larger capillary (75-\(\mu\text{m}\) I.D) and a much smaller focusing voltage than this experiment, it is quite perceivable that the reduction of the first term in our experiment will approach \(10^2\).
Combining the effects of narrower diameter capillary with applied focusing voltage, peak height during PM can be reduced by several hundred folds. This has significant implications, as the increase in mobilization velocity will no longer adversely impact resolution.

These predictions are corroborated experimentally: resolution in PCM is comparable to those in CM and PM as shown in Table 3.1, with the exception of pI 5.5 peak. However, considering that the CAs are not reproducibly homogeneous (Section 2.3.2), it is premature to make definite statements about resolution without replicate experiments. It is also prudent to be cautious with the calculated resolution, since the peaks are too far apart to give an accurate representation of the true resolving power. Nonetheless, comparable resolutions in all three mobilization methods seem to indicate no negative impact on resolution during PCM. With approximately 33% decrease in analysis time using PCM, it is recommended as an improvement over CM or PM.

### 3.3.3 Anti-convective focusing media

Neutral hydrophilic coating used in this experiment serves two purposes: to minimize electroosmotic flow (EOF) and protein adsorption. In addition, anti-convective media such as cIEF gel or glycerol were implemented in the sample mixture to further reduce EOF and protein adsorption while minimizing the diffusion coefficient by increasing solution viscosity. Glycerol in particular has been demonstrated to be an effective anti-convective medium, even during cIEF with uncoated capillaries. Furthermore, its compatibility with mass spectrometry (MS) makes it preferable over the polymeric cIEF gel.
To evaluate the merits of adding glycerol to the sample mixture, cIEF with varying concentrations of glycerol was conducted. Chemical mobilization was unsuccessful with 30% v/v glycerol as evidenced by the lack of current during mobilization. Stacked electropherograms with 5 to 20% v/v glycerol are found in Figure 3.3.

![Electropherograms of five peptide pI standards](image)

Figure 3.3 Electropherograms of five peptide pI standards (pI 10.0, 9.5, 7.0, 5.5, 4.1) in varying concentrations of glycerol. Pressure-assisted chemical mobilization started at 12 minutes with 1 psi applied at the inlet. A: 5% (offset by 0.03 Au); B: 10% (offset by 0.02 Au); C: 15% (offset by 0.01 Au); and D: 20% v/v glycerol in water as separation media.

The most visible benefit to reducing glycerol concentration in Figure 3.3 is the significant decrease in analysis time. With 5% v/v glycerol in the sample mixture, the entire analysis can be completed in 40 minutes, including rinsing time. This is accomplished in two ways in PCM: increase in electrophoretic mobility and solution fluidity. Electrophoretic
mobility, $\mu_{ep}$ is inversely proportional to solution viscosity according to the following equation:

$$\mu_{ep} = \frac{q}{6\pi\eta R}$$  \hspace{1cm} (Eq. 3.5)

where $q$ is the charge of the ion, $\eta$ is the solution viscosity, and $R$ is the solvated ionic radius. Increase in viscosity heightens the drag force, which opposes the electrophoretic movement caused by the electrostatic force. This phenomenon applies to counterions in the mobilizer solution which titrate the pH gradient, and to analyte ions which gain temporary positive charges during mobilization. Thus, during any form of chemical mobilization, reduction in solution viscosity will accelerate mobilization. Additionally, reduction in viscosity can improve solution fluidity by minimizing sheer stress.

On the other hand, improving analysis time by adjusting solution viscosity implies foregoing the benefits of anti-convective separation medium: namely, improved protein solubility, reduced wall adsorption, and enhanced focusing due to decrease in diffusion coefficient. With small peptide pI markers used in this experiment, protein precipitation and adsorption are not of a great concern. However, increase in diffusion coefficient with less viscous separation medium can adversely affect resolution and sensitivity. To study these effects, peak areas of the pI standards as a function of glycerol concentration are plotted in Table 3.2.

Peak area, as a measure of sensitivity, increases with glycerol concentration in the sample mixture with good linearity in all five pI standards ($R^2 > 0.975$). In other words, improvement of analysis time comes at the direct cost of analyte sensitivity when glycerol
concentration is the sole variable. If the total analysis time is to be considered, the gain in speed may seem disproportionately weak compared to the cost in sensitivity. However, the decision is left to the investigator, depending on the application. If analysis time is of crucial importance in fields such as high-throughput biomarker screening, sacrifice in sensitivity may be justified. This is especially relevant if a more sensitive detector such as laser-induced fluorescence or mass spectrometry is available. Alternatively, if the analysis involves low-abundance proteins, slight increase in analysis time may be worthwhile.

Table 3.2 Peak areas of five pI standards as a function of glycerol concentration in the sample mixture.

<table>
<thead>
<tr>
<th>pI Standards</th>
<th>20% Glycerol</th>
<th>15% Glycerol</th>
<th>10% Glycerol</th>
<th>5% Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0</td>
<td>10772</td>
<td>9756</td>
<td>8305</td>
<td>6818</td>
</tr>
<tr>
<td>9.5</td>
<td>9172</td>
<td>7475</td>
<td>6670</td>
<td>5249</td>
</tr>
<tr>
<td>7.0</td>
<td>40245</td>
<td>34370</td>
<td>29022</td>
<td>26327</td>
</tr>
<tr>
<td>5.5</td>
<td>42392</td>
<td>36338</td>
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<td>25273</td>
</tr>
<tr>
<td>4.1</td>
<td>33567</td>
<td>30104</td>
<td>24591</td>
<td>18423</td>
</tr>
</tbody>
</table>

3.3.4 Focusing time

The length of focusing time is another parameter that can be optimized to improve analysis time. The start of mobilization is often determined empirically by observing the separation current during cIEF. When the current reaches a low plateau, it is assumed that there is minimal ion movement as the proteins become focused at their pIs along the pH gradient. Lengthening focusing time can improve sensitivity by providing sufficient time for residual protein molecules to find their pIs. However, if focusing is extended too long, it can cause protein precipitation or adsorption, resulting in reduced sensitivity. To compare the loss of sensitivity when focusing time is reduced, cIEF experiments with 7 and 12 minutes of focusing were conducted. Their peak areas are found in Table 3.3.
As expected, shorter focusing time resulted in reduced sensitivity. However, if the application puts a greater emphasis on rapid analysis than on sensitivity, 7-minute focusing is sufficient for high-resolution separation of peptide pI markers. The total analysis time with 7-minute focusing was 20 minutes. Considering that this was chemical mobilization, even shorter analysis time is possible with PCM. This is the shortest cIEF-UV analysis with a 50-cm capillary to the best of our knowledge. Since our CE instrument can support down to 30-cm capillaries for UV detection, 10-minute total analysis of cIEF-UV is within the realm of probability.

### 3.3.5 Compatibility with mass spectrometry

One of the goals of this investigation was to evaluate and optimize 30-μm neutral capillary for future application with mass spectrometry. Optimization of cIEF analysis in this study enables smooth transition from UV to MS as a detector, in addition to shortening analysis time. Elimination of arginine as cathodic spacer will safeguard mass spectrometer from being bombarded with high concentration of arginine, which could contaminate the sampling orifice and saturate the mass analyzer. Pressure-assisted chemical mobilization is also ideal for coupling with MS, as its increased flow rate can introduce concentrated amount of analytes without loss of resolution while reducing the gap in flow rate that is prevalent in chemical mobilization. Possible incompatibility of cIEF gel with MS during
PCM is negated by replacing it with glycerol-water solution, which has been used successfully with MS in our lab\textsuperscript{48}. Changing other parameters such as glycerol concentration and focusing time does not present foreseeable difficulty with MS detector. They can be adjusted, depending on the nature of the sample, for optimum sensitivity or for shortest analysis time.

While results of this investigation largely favour 30-μm capillary over 50-μm capillary in terms of MS compatibility and analysis time, one downside of using 30-μm capillary is the loss of capillary volume. With almost one-third reduction in volume, additional dilution by the sheath liquid during electrospray ionization may render some low-abundance analytes undetectable. Like adjusting glycerol concentration and focusing time, choosing a suitable diameter of the capillary is a decision that must be made for each particular application.

3.4 Conclusions

Capillary isoelectric focusing with 30-μm neutral coating capillary was successfully optimized by adjusting cathodic spacer concentration, mobilization method, concentration of anti-convective separation medium, and length of focusing time. Fully optimized cIEF with 30-μm capillary instead of 50-μm capillary has many benefits in terms of analysis time as well as coupling with mass spectrometer as a detector. With 30-μm capillary, arginine as a cathodic spacer was eliminated without any loss of high pI analytes. This will protect the mass spectrometer from high concentration arginine and also ensure optimum analysis time. Pressure-assisted chemical mobilization can replace chemical or pressure mobilization to shorten analysis time, without adversely affecting resolution. Reducing
concentration of anti-convective separation medium and focusing time can also shorten analysis time, but it is at the direct cost of analyte sensitivity.

One significant disadvantage of using a 30-μm capillary is the reduced sensitivity. With a UV detector, shorter path length would result in lower absorbance, thus rendering some low-abundance analytes undetectable. With a MS detector, lower capillary volume would produce fewer analyte ions, also resulting in difficulty observing low-abundance analytes. However, despite the loss of sensitivity, effective and rapid cIEF can make a big difference when analyzing a large number of samples. With all optimizations, a cIEF run could be completed within 15 minutes without rinsing, which is the fastest cIEF analysis to our knowledge. For experiments in which analysis time is of utmost concern, 30-μm neutral capillary is an excellent choice for rapid, reproducible, high-resolution, and MS-compatible capillary isoelectric focusing.
Chapter 4: CONCLUSIONS
4.1 REALIZATION OF RESEARCH OBJECTIVES

For biological analyses, fast and reproducible separation methods are in high demand. Capillary isoelectric focusing is a method of separation that has unparalleled resolving power for amphoteric analytes. Furthermore, it can provide important insight into the identity and structure of proteins using their predicted isoelectric points. Our goal in this endeavour was to bring capillary isoelectric focusing to the forefront of modern bioanalysis by significantly reducing analysis time. We were able to reduce analysis time to a fraction of the 60-minute analysis observed with a 50-µm capillary in Chapter 2 by employing a narrow-diameter capillary and optimizing several experimental parameters. In addition, study of reproducibility and effect of adjusting these parameters lent us insights into inner workings of the technique.

4.1.1 Reproducibility of capillary isoelectric focusing

Reproducibility of cIEF is a rather elusive subject. It may appear to be poor at the first glance of the results as migration times vary greatly, up to 8.0% RSD. However, upon close inspection, isoelectric points of myoglobin isoforms are precisely and accurately determined during the lifespan of the neutral coating. Variations in migration times stem from heterogeneity of carrier ampholytes, which is an inevitable by-product of the chaotic synthetic process. Linearity of pH gradient is also debatable, as it is affected by capillary temperature, presence of salt, and high concentration of analytes and standards. Fortunately, the poor reproducibility of pH gradient can be remedied by using pI markers as cIEF-equivalent of internal standards. Although linearity of the entire pH gradient is difficult to ascertain, linearity between two neighbouring pI markers can be assumed as a
good approximation. By using two pI markers that bracket analyte of interest, pI of an unknown protein can be determined accurately and precisely. Reproducibility of calculated pI improves if the two markers are as close to the analyte as possible. Therefore, excellent reproducibility in cIEF can be achieved by choosing appropriate pI standards.

There are a few additional observations about stability of Beckman Coulter neutral coating that should be mentioned. Suppression of electroosmotic flow by the coating was outstanding for 50 consecutive runs. Even after it began to show signs of deterioration, electroosmotic mobility remained relatively low, at $2.2 \times 10^{-5}$ cm$^2$ V$^{-1}$ s$^{-1}$. However, complete prevention of analyte adsorption was not achieved and resulted in steady increase of migration times. Unfortunately, high concentration of neutral protein at its pI makes protein adsorption unavoidable, as demonstrated by Graf and Wätzig$^{43}$. To overcome these effects, the capillary was conditioned through extended rinsing, which shortened the lifespan of the coating. While there are reports of more robust coatings with superior longevity$^{22-23}$, they are laborious to prepare and often require additional instrumentation. Beckman Coulter neutral coating offers a tempting alternative by providing exceptional EOF suppression and sufficient protection against analyte adsorption. We have been collaborating with scientists in industry to develop a new generation of coatings for cIEF. The results have been promising, but will not be discussed in this document.

4.1.2 Optimization for rapid capillary isoelectric focusing

In many separation methodologies, speed and quality of analysis are two arms of a balance. Fortunately, employing 30-μm capillary is in large part an exception to the rule and optimization for speed can be accomplished with minimal sacrifice to the quality of
analysis. Substantial difference in volume between separation space and catholyte reservoir enabled elimination of cathodic spacer that was previously impossible on a larger-diameter capillary. More importantly, band broadening due to laminar flow by pressure mobilization was minimized with a narrow capillary and diligent application of electric field. This reduced analysis time by a third without any detectable loss in resolution and sensitivity. Further adjustments of experimental parameters such as focusing time and separation medium shortened analysis time by another half, although these came at the cost of sensitivity. Capillary isoelectric focusing is an innately sensitive technique, owing to the double focusing nature of separation. As such, sensitivity may be a worthwhile sacrifice for improving analysis time. With all optimizations, total analysis time of full-range pH gradient is reduced from 60 to 15 minutes, which is the shortest to the best of our knowledge. Further reduction is possible with narrow-range pH gradient and shorter capillary for specific analytes.

4.2 Future Research Directions

4.2.1 Coupling with mass spectrometry

Mass spectrometer is robust, sensitive, reproducible, and informative. Moreover, it is an analytical tool in its own right, as it adds a new dimension of separation by mass-to-charge ratio. Lastly, analytes can be further scrutinized by tandem MS to reveal structure as well as intermolecular interactivity. Optimization of cIEF in this thesis was conducted with explicit purpose of coupling with MS in the near future. Successful coupling of cIEF-MS has already been demonstrated in our group with a novel sheath-liquid interface\textsuperscript{48}. Elimination of cathodic spacer and addition of pressure mobilization in this thesis are especially well
suited for coupling with MS. Furthermore, the smaller mobilizer reservoir in our cIEF-ESI interface compared to catholyte vial in UV experiments may lessen the steepening of pH gradient at cathode and result in even faster mobilization. An online cIEF-MS with short analysis time in the scale that has been demonstrated in this thesis will be a promising analytical tool for complex biological samples.

4.2.2 Rapid targeted screening

Many applications of cIEF do not require a full range of pH gradient used in this thesis. For narrow pH range analyses, a shorter capillary with a narrower range of carrier ampholytes can be employed for high-throughput screening. Applications that are especially suitable for this approach include separating deamidated species of recombinant DNA products$^{84}$ and screening glycated hemoglobin for diabetes$^{85}$. Optimized methods developed in this thesis may be applied using a short capillary to achieve an even shorter analysis time in the range of several minutes. Loss of resolution through reduced separation space can be minimized by using narrow pH range carrier ampholytes. Successful adaptation of cIEF to high-throughput screening will bring it one step closer to the forefront of current bioanalysis.

4.3 Concluding Remarks

In the words of Pier Giorgio Righetti$^{84}$, “things with cIEF are not as smooth as here presented.” It has come a long way from the days of Svensson, when he searched endlessly for magical compounds that would spontaneously form a smooth pH gradient under electric field. Since then, the exceptional resolving power of cIEF has been demonstrated many times$^{85-87}$. The capability for automation and online coupling with MS is also very
promising indeed. Despite these merits, cIEF has largely been overshadowed by HPLC and UPLC in bioanalytical field. This is in part no surprise, as liquid chromatography has a much longer history and has a ubiquitous presence in almost every bioanalytical laboratory. More importantly, current analytical capabilities of HPLC and UPLC are demonstrably unparalleled, as they share many of the strengths of cIEF in addition to short analysis time and a large selection of stationary and mobile phases to suit almost any application.

Notwithstanding the small share of bioanalytical field that cIEF holds currently, we are hopeful for its future. Its capability for reproducible, sensitive, automated, and fast separation has been demonstrated in this thesis. Additionally, insights into inner workings of experimental parameters have unfolded through computer simulation and experimental studies. Many promising methods for coupling with MS have also emerged in the past decade. Capillary isoelectric focusing is a unique dimension of separation that can be incorporated to existing analytical methodologies. Its ability to separate protein isoforms and modifications while providing information on the analytes’ isoelectric points makes it a great addition to any analytical chemist’s arsenal. Our work to significantly shorten analysis time of cIEF can bring us one step closer to realizing the full potential of this technique.
Bibliography

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